Quantification of Jasmonic Acid by SPME in Tomato Plants Stressed by Ozone

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Jasmonates are signalling molecules induced in plants as a response to various biotic and/or abiotic stresses. As ozone is known to activate defense responses in plants, we have monitored the concentration of jasmonic acid in tomato leaves during and after an acute exposure to this abiotic elicitor. In this experiment, we observed that the maximum induction of jasmonic acid in O_3-fumigated plants occurred 9 h after the end of treatment and the concentration of jasmonic acid in stressed plants increased 13-fold. However, the level of endogenous methyl-jasmonate was constant during the observed period. The extraction and quantification of jasmonic acid as its methyl ester was performed by headspace-solid-phase microextraction (or HS-SPME) in combination with GC−FID and GC−MS. The sensitivity (LOD = 2 ng/g) of this method permitted the detection and quantification of jasmonic acid present in plant tissues at very low concentrations.

KEYWORDS: Tomato; jasmonic acid; ozone; HS-SPME

INTRODUCTION

Plants respond to several environmental stresses such as pathogen attack, mechanical or herbivorous insect-driven wounding, and the presence of pollutants by the synthesis of secondary metabolites that mediate interplant communication for defense responses. Among these signalling molecules, three are considered the major regulators of plant defense responses: salicylic acid, jasmonic acid, and ethylene (*1*, *2*). It is known that the role of these three signalling molecules is not independent, but an antagonism between the pathways is sometimes possible. Several lines of evidence support the hypothesis that a negative feedback regulation between the signalling pathways is possible (*3*, *4*).

The role of salicylic acid in influencing plant resistance to pathogens (aphids, bacteria, fungi) is well documented (*5*), whereas jasmonic acid is commonly believed to play an important role in plant responses to insect herbivores and abiotic stress. In fact, wounding (*6*, *7*) and pathogen elicitors can induce accumulation of jasmonic acid, which is responsible for the induction of a specific gene induced by wounding, the proteinase inhibitor (*PinII*) (*6*).

The biosynthetic pathway of jasmonates (jasmonic acid and its derivatives) in plants has been intensively studied and well explained. The soluble jasmonic acid acts as a direct defense to herbivores or others injuries, whereas the volatile form,

methyl jasmonate, represents a plant signal molecule, which serves as an indirect defense mechanism. Methyl jasmonate is synthesized from jasmonic acid by carboxyl methyltransferase (EC 2.1.1.141), and the methyl ester stimulates the production of jasmonic acid in surrounding plants even though they are not under stress. There is some evidence for the activation of jasmonate methyltransferase in distal leaves of *Arabidopsis* by wounding or exogenous methyl jasmonate application (*8*), and methyl jasmonate has become a strong candidate for a role in systemic signalling. This compound could diffuse to distal part of plants via vapor phase (*9*) or by intracellular migration, possibly through the phloem (*10*), whereas jasmonic acid might not be able to move across the cellular membrane without a specific carrier because of its acidic nature (*11*). The levels of jasmonic acid in plants vary with developmental stage, organs, and species. Results obtained by several groups indicate that plants can have a jasmonic acid concentration between 10 and 1500 ng/g depending on the factors described above and physiological conditions (*12*).

Environmental stress such as low temperature, intense light, UV irradiation, and ozone can influence jasmonic acid concentration in plants. One of the main components of photochemical pollutants is ozone (O_3). It has a strong oxidative potential ($E°$ = 2.07 V) and represents a serious problem in many countries because it can damage animals and plants. Tropospheric ozone is believed to cause significant damage and loss of productivity in native and crop plants (*13*, *14*). With respect to animals, plants are more susceptible to ozone, and sensitive species can show damage after a short exposure to even low concentrations (3−4 h, 50−60 ppb). This poses a problem because O_3 concentrations in industrial areas worldwide can vary between 100 and 400

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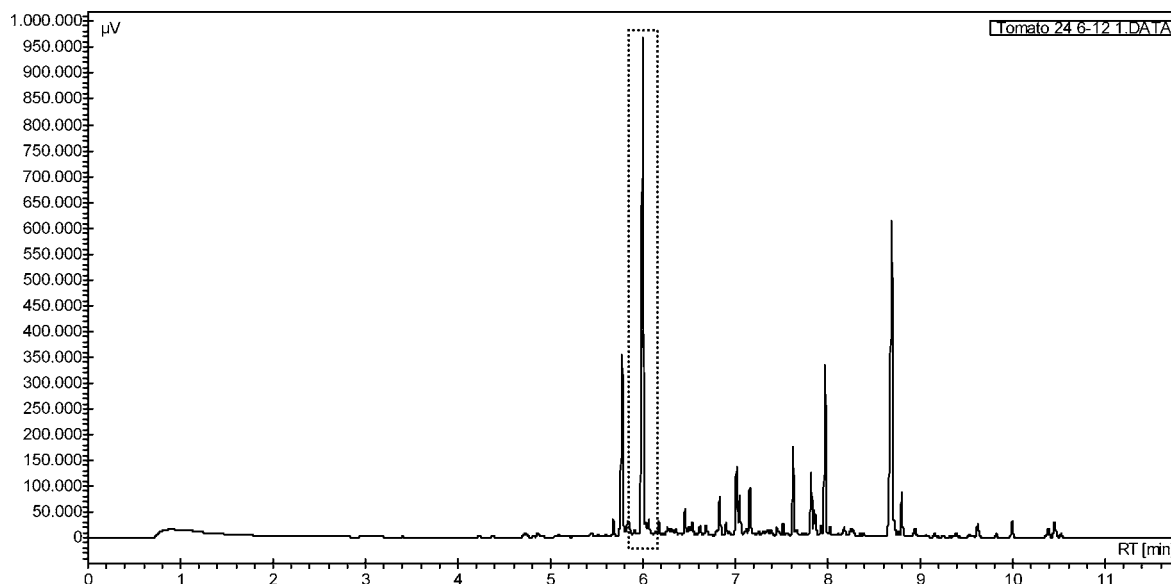‡ Department of Plant Biology and Agro-environmental and Animal Biotechnology.

Figure 1. Representative GC chromatogram of jasmonic acid (as methyl jasmonate) accumulated in leaves of tomato after ozone exposure. The peak corresponding to the analyte is marked.

ppb (*14*). Ozone diffuses into plants via stomata, and so the green tissues are the major target-sites of its action. In leaves, O_3 leads to an oxidative burst, massive, rapid, and transient activation of oxidative metabolism (*15*). The acute exposure to ozone could cause a suicide program in cells similar to that shown after pathogen infection. This phenomenon is referred to as cell death around the invasion site and as a transcriptional activation of defense genes in both challenged and surrounding cells.

Because jasmonic acid is commonly regarded as a protective compound that may control plant lesions upon oxidative stresses, this study was done to investigate the changes of jasmonic acid concentrations after whole tomato plants were exposed to acute O_3 treatment in comparison with the untreated plants. For this study, a HS-SPME methodology for the extraction of jasmonic acid as methyl jasmonate from plant samples was optimized evaluating the main parameters that can affect the extraction efficiency and selectivity of the SPME technique.

MATERIALS AND METHODS

Chemicals. Methyl jasmonate analytical standard (95%) was purchased from Sigma-Aldrich (St. Louis). Silicon dioxide was purchased from Riedel-de-Haën (Seelze, Germany). NaCl and EtOH were purchased from BDH (VWR International, England). Jasmonic acid was prepared from methyl jasmonate according to Creelman et al. (*16*). Diazomethane was prepared according to the procedure described by Zadra (*17*).

SPME Materials. The manual SPME fiber holder and SPME fibers (PDMS, 100 μm film thickness) were purchased from Supelco (Bellefonte, PA). The new fibers were conditioned before use according to manufacturer's instructions, 1 h in the GC-injector at 250 °C.

Optimization of the HS-SPME Method. *Extraction Temperature.* Measurements were performed on standard solutions containing a range of 10−1000 ng/g of analyte. A sample temperature ranging from 25 to 60 °C was examined, and dependence of the area on the temperature of sample was studied.

Exposure Time. After sample temperature optimization, the exposure time was studied in standard solutions. The samples were magnetically stirred, and the SPME fiber was exposed to the headspace for periods of time ranging from 5 to 90 min. The extracted analytes were immediately chromatographed. Temperature was kept at 60 °C during the experiment, which was performed in triplicate.

Effect of Ionic Strength. The ionic strength of solution was modified by addition of NaCl on standard solutions of analyte. Experiments replacing the distilled water as sample media with aqueous NaCl solution with concentrations ranging from 0% to 30% were conducted. The temperature was 60 °C, and the extraction time was 30 min. **Figure 1** shows an example of the SPME-GC chromatogram under optimized conditions.

Plant Material and Ozone Treatment. Tomato seeds (*Lycopersicon esculentum* Mill.) cv New Yorker were kindly provided by Prof. G. P. Soressi (Dept. of Agrobiology and Agrochemistry, Univ. della Tuscia, Viterbo, Italy). Tomato plants were grown under controlled conditions (20 ± 1 °C, 85 ± 5% RH, 500 μmol photon flux density/m^2/s) and were sampled at the growth stage of fourth true leaf .

O_3 fumigation was performed according to Bernardi et al. (*18*). Plants pre-adapted to the chamber conditions for 48 h were exposed to an acute fumigation with 200 ppb O_3 for 5 h and left to recover in charcoal-filtered air. Leaves were sampled periodically at 0, 1, 3, 5 h (time during the exposure to O_3), and 8, 11, 14, 17 h (time after the end of exposure to O_3). Control untreated plants were kept in charcoal-filtered air chambers under the same growth conditions.

Extraction of Jasmonic Acid from Tomato Leaves. Frozen plant material (0.3−0.5 g of fresh weight) was pulverized with liquid nitrogen and silicon dioxide using a pestle and mortar. An aliquot of methanol (1:2, w/v) was added, and the mixture was centrifuged at 10 000*g* for 15 min at 4 °C. The supernatant was collected in a 4 mL glass vial, and the solvent was evaporated under N_2 at ambient temperature.

2 mL of ethereal diazomethane was added to the dried sample for the derivatization reaction, and after 30 min the reaction was stopped under a gentle N_2 stream. 1 mL of 30% w/v NaCl solution was added to the dried sample in a vial sealed with a silicon septum and containing a stir bar. Extraction of methyl jasmonate was carried out by headspace exposure of the fiber over the aqueous sample under stirring at 60 °C for 30 min. The temperature of water samples was controlled by means of a device with Pt thermometer with a precision of ±0.01 °C (Julabo Labortechnik GmbH, Seelbach, Germany).

Blank analyses were carried out by exposure of the fiber to the saline solution.

GC Analysis. Analyses were performed with a Varian CP-3800 gas chromatograph (Varian Inc., Palo Alto, CA) equipped with a 1177 split/splitless injector, a 30 m × 0.25 mm i.d., 0.25 μm, CP-Sil8CB capillary column (Varian), a FID detector, and Galaxie Workstation software (Varian Inc.). Desorption of the PDMS-SPME fiber was made directly into the injector port for 5 min at 250 °C in splitless mode. The injector split/splitless program mode was: 0−5 min splitless; 5.01−5.75 min at 1:50 split ratio. The column oven was programmed as follows: 60

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°C (1 min) to 280 °C (2 min) at 25 °C/min. The temperatures of the injector port and detector were 250 and 280 °C, respectively. Helium was used as carrier gas, and its pressure was maintained constant at 10.0 psi (1 mL/min).

GC−MS Analysis. Analyses were made to confirm the efficacy of the methylation procedure. A Varian Star 3400 gas chromatograph equipped with a split-splitless injector was combined by direct coupling to a Varian Saturn II mass spectrometer, operating in the electron impact mode (EI), equipped with a multiple-ion detector. A 30 m × 0.32 mm i.d., 0.25 μm ZB-5 capillary column (Phenomenex, Torrance) was used. The chromatographic conditions were as follows: the temperature was programmed from 60 °C (1 min) to 280 °C (9 min) at 25 °C/min. The carrier gas was helium at the flow rate of 1 mL/min; the temperatures of the injector port and of the ion source were 250 and 280 °C, respectively; and the emission current was 10 μA. The ions to be taken into consideration in confirmation of jasmonic acid (as methyl jasmonate) were: [M$^+$] m/z 224 (90), 202 (35), 193 (32), 187 (20), 177 (28), 151 (100), 135 (74), 117 (15), 107 (50), 95 (60), 83 (87), 67 (52), 55 (54).

Statistical Analysis. Statistics calculations on the data obtained from SPME fiber analyses were performed via the ANOVA two-way test after the logarithmic transformation of the results.

RESULTS AND DISCUSSION

The quantification of jasmonic acid in fully expanded leaves is difficult because of its low level and instability during the purification steps (*19*, *20*). The common protocols to determine jasmonic acid require a long time for sample preparation and purification, and a large amount of organic solvents are required in several steps of the analytical method.

An alternative sample preparation technique with increasing success is solid-phase microextraction (SPME). This technique is based on the specific sorption of analytes by the film coating of a silica fiber support, allowing extraction and concentration in a single step without long and laborious purification steps. Although a very small amount of analyte is extracted, the SPME technique avoids the use of large amounts of organic solvents, and the losses that could occur during the extraction, concentration, and cleanup steps of traditional analytical methods are reduced. In fact, the LODs (2 ng/g) obtained with the HS-SPME technique applied to this experiment are due to low levels of losses that could occur during sample preparation.

SPME Procedure. In this study, we have used headspace-SPME for sampling JA-derivatized for several reasons: the methyl ester is a volatile compound; the equilibrium is attained more rapidly in headspace SPME than in immersion mode; the analyte can diffuse more quickly to the coating on the fiber; and the headspace mode is ideal for minimizing interferences with analysis and can prolong the lifetime of the SPME fiber.

The efficiency of SPME is affected by a number of variables; therefore, preliminary studies were carried out to determine the sampling time, ion strength (% NaCl), and temperature for a better efficiency of the whole extraction. Optimization of the absorption time of jasmonic acid-derivatized on SPME-PDMS fiber was investigated by triplicate extractions of a methyl jasmonate from aqueous standard solutions over a period from 5 to 90 min. Plotting area versus exposure time (**Figure 2A**), the optimal exposure time was 30 min.

The ionic strength of the solution influences the equilibrium partitioning between liquid phase and headspace. To establish the optimal salt percentage of aqueous solution, the addition of % different amounts of NaCl to standard solution of methyl jasmonate was studied. The amount of analyte adsorbed at any concentration was plotted against % NaCl (w/v). The maximum amount of analyte adsorbed was found at an optimal concentration of 30% NaCl (**Figure 2B**). No interfering peaks at the same
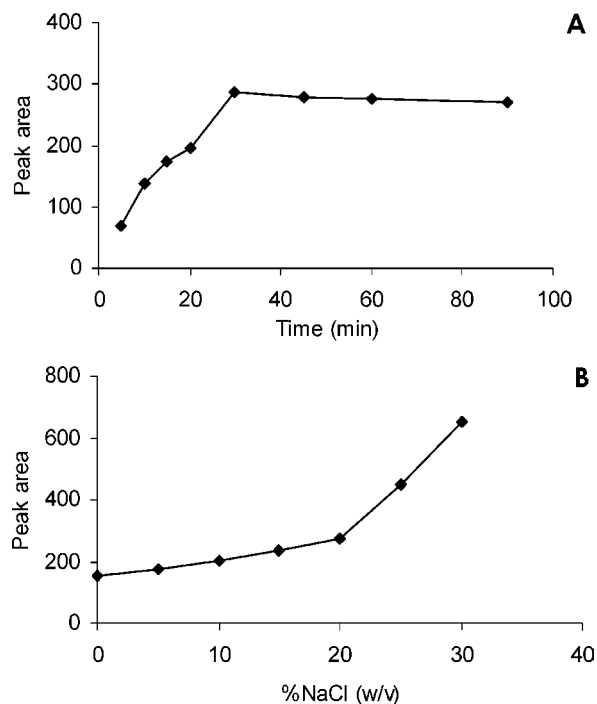
Figure 2. (**A**) Influence of headspace exposition time in the efficiency of methyl jasmonate (5 ppb) extraction with PDMS-SPME fiber. The data show the averages of four different experiments. (**B**) Influence of % NaCl on the efficiency of methyl jasmonate (5 ppb) extraction with PDMS-SPME fiber. The data represent the mean of four independent experiments.

methyl jasmonate retention time were recorded when blank analyses were carried out with fiber exposed to ambient air and 30% NaCl solutions. In fact, to avoid contamination problems of memory effects during the analysis of samples, fibers were additionally heated at 250 °C for 15 min at the end of chromatographic analysis.

To optimize the amount of CH$_2$N$_2$ necessary for derivatization of JA in plant samples, jasmonic acid standard solutions (0.1−10 ng/μL) were added with increasing concentrations of ethereal diazomethane, and the reaction was monitored by GC−MS analyses.

To determine the recovery of free jasmonic acid in the method developed, known amounts of jasmonic acid were added to plant matrices prior to extraction. Spiked plant samples were subjected to extraction and quantification. The data obtained from these injections were plotted against the amount of jasmonic acid standard added (**Table 1**). A good linearity for the proposed method was observed. The amounts of endogenous methyl jasmonate found were then subtracted to calculate the concentration of jasmonic acid in controls and O$_3$-fumigated plants. The quantity of analyte was determined as usual from the ratio of the amount (C_e) found to the sum of the amount (C_{ad}) added plus that originally present in the matrix (C_0). Because C_e and C_0 are unknown, a linear relationship for methyl jasmonate was established.

The analytical method was simple, fast, and reliable. The limit of detection (LOD) for jasmonic acid as methyl jasmonate was calculated as 2 ng/g, the same results as obtained by Meyer et al. (*19*) in *Arabidopsis*. This level of sensitivity was also sufficient to calculate the concentration of endogenous methyl jasmonate in healthy tomato plants.

Quantification of Jasmonic Acid in Fumigated Tomato Plants. To estimate the levels of endogenous methyl jasmonate, plant material (treated ozone plants and control plants) was extracted by SPME without the derivatization step. It was

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Table 1. Extraction Recovery (± Standard Error) and Concentration (± Standard Error) of Jasmonic Acid in Plant Samples Spiked with Known Amounts of Jasmonic Acid Standard[a]

jasmonic acid added (ng)	recovery (×100)	jasmonic acid concentration (ng/g FW)
0		8.0 ± 0.1
2	88.4 ± 0.8	8.8 ± 0.1
5	84.2 ± 0.6	10.9 ± 0.1
10	90.1 ± 0.4	16.2 ± 0.1
20	83.0 ± 0.6	23.4 ± 0.2
50	90.1 ± 1.0	52.2 ± 0.6
100	89.2 ± 0.4	96.3 ± 0.5
200	87.5 ± 0.6	182.0 ± 1.3
500	90.4 ± 1.0	459.4 ± 5.2
1000	85.1 ± 1.4	857.8 ± 14.5

[a] The data represent the average of four replicates.
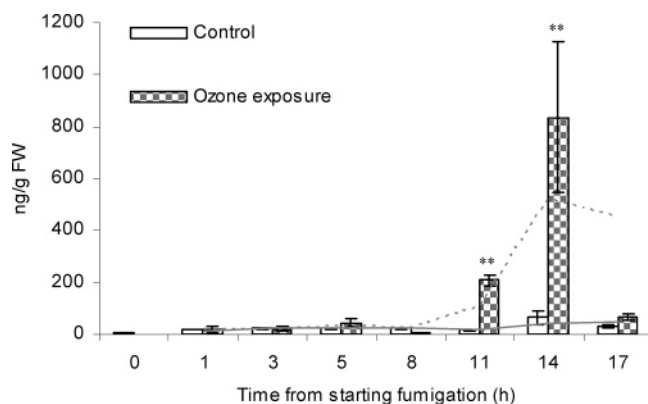
Figure 3. Jasmonic acid concentrations in tomato leaves during and after ozone exposure. Open bars, plants left in charcoal filtered air; filled bars, plants treated with O_3, 200 ppb for 5 h. The data are reported as ng/g FW (**: $P = 0.01$).

possible to estimate only the endogenous levels of methyl jasmonate, because the acidic form was not able to pass into the headspace and thus was not adsorbed by the fiber. The results for the quantification of jasmonic acid (as methyl jasmonate) concentrations in tomato plants are shown in **Figure 3**. An increase in jasmonic acid level was detected after 6 h from the end of fumigation, and the maximum accumulation was observed at 9 h from the end of fumigation. Jasmonic acid decreased after 12 h of the recovery time, with a level similar to that revealed in control plants. Thus, the tomato plants exposed to ozone showed an increased jasmonic acid concentration by 13.0-fold as compared to control plants. Statistical analysis of the results has shown that the differences were due to treatment, time, and treatment × time ($P = 0.01$). During our experiment, no differences in the levels of endogenous methyl jasmonate were detected in ozone-damaged plants (data not shown).

In agreement with other authors (20, 21), our analyses clearly show that jasmonic acid is involved in the response to ozone treatment of tomato plants. Rao et al. (22) have shown that jasmonic acid level in *Arabidopsis* plants increased after fumigation with ozone. Moreover, the present report shows a method to estimate the concentrations of a phytohormone, which is believed to play a central role in plant defense responses. The method for extraction and quantification of jasmonic acid presented here has some advantages with respect to the methods currently used. It is faster, reliable with a low amount of plant material, and does not require the use of organic solvents.

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